Studies on fungi associated with *Tomicus piniperda* L. in Finland.

Ximena Silva
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University of Helsinki
Department of Forest Sciences
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HELSINGIN YLIOPISTO - HELSINGFORS UNIVERSITET - UNIVERSITY OF HELSINKI

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Tekijä ¾ Författare ¾ Author

Ximena Silva

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Tiivistelmä ¾ Referat ¾ Abstract

The Scots pine bark beetle, *Tomicus piniperda* is a secondary colonizer of pine and other conifers. It is a native species from Europe and Asia that was recently introduced in North America. Although it is necessary to understand this insect's interactions with other organisms, few studies have focussed on its fungal associates. This study focused on the effect of latitude in the occurrence of fungi associated with *T. piniperda*. *T. piniperda* were collected from *Pinus sylvestris* in Northern (Rovaniemi) and Southern (Hyytiälä) Finland. Both endo- and epi- mycota were isolated. The fungi were identified using a combination of morphological features and molecular data. The results revealed a great diversity of fungi species associated with *T. piniperda*, with a total of 3073 isolates representing 23 species. The most frequently isolated fungi in the bark beetles from Northern Finland were *Beauvaria bassiana, Kuraishia* sp. and *Penicillium* sp. whereas *P. brevicompactum* and *Mortierella* sp. were mostly observed in the South. *Ophiostoma canum* and *O. minus* were also observed. The number of isolates per insect in the north was 2.83 epi- and 2.38 for endo-mycota fungus. In the south, the number of isolates per insect was 4.1 for epi- and 3.5 for endo-mycota. Statistical analysis indicated that there was significant differences in fungal populations associated with the beetles in Southern and Northern Finland. There was however no significant difference between the epi- and endo-mycota fungal populations. The highest richness and diversity of the fungal species was observed in the South. However, the overall fungal diversity index analysis revealed that the mycobiota was undersampled.

Avainsanat ¾ Nyckelord ¾ Keywords

Tomicus piniperda, insect, fungus, Finland, DNA analysis*, Ophiostoma*

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Thesis supervisor: Prof. Frederick Asiegbu, Assistant supervisor: Eeva Terhonen

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Tiivistelmä ¾ Referat ¾ Abstract

Pystynävertäjä (Tomicus piniperda L) on mäntyjen ja muiden havupuiden tuholainen. Se kuuluu Euraasian alkuperäiseen lajistoon ja on hiljattain levinnyt myös Pohjois-Amerikkaan. Huolimatta pystynävertäjän yleisyydestä ja merkityksestä, hyönteisen sieniosakkaat tunnetaan huonosti. Tässä työssä tutkittiin maantieteellisen sijainnin vaikutusta pystynävertäjän kuljettamien sienten monimuotoisuuteen. Hyönteisiä kerättiin sekä Etelä-Suomesta (Hyytiälä, Juupajoki) että Pohjois-Suomesta (Rovaniemen ympäristö).

Sieniviljelmiä eristettiin hyönteisten sisältä (endo-entomofaagiset sienet) ja pinnalta (epi-entomofaagiset). Sienet tunnistettiin sekä morfologisesti että molekulaarisin menetelmin.

Tulokset osoittavat, että pystynäveräjään on assosioitunut runsaasti sienilajeja (23 kpl) ja yksilöitä (3073 sienikantaa). Yleisimmät sienet Pohjois-Suomessa olivat Beauvaria bassiana, Kuraishia sp. ja Penicillium sp., kun taas P. brevicompactum ja Mortierella sp. olivat yleisimpiä Etelä-Suomessa. Myös Ophiostoma canum and O. minus-lajeja havaittiin. Pohjois-Suomesta hyönteisten pinnalta eristettiin keskimäärin 2,83 sienilajia/hyönteinen ja hyönteisten sisältä 2,38 lajia/hyönteinen. Etelä-Suomessa yksittäisten hyönteisten pinnalta havittiin keskimäärin 4,1 sienilajia ja sisältä 3,5 lajia/hyönteinen.

Pohjois- ja Etelä-suomen sieniyhteisöt erosivat toisistaan tilastollisesti merkitsevästi. Hyönteisten sisällä ja pinnalla esiintyvien sienten välillä ei ollut merkitseviä eroja. Suurimmat lajimäärät ja diversiteetti havaittiin Etelä-Suomesta. Monimuotoisuusindeksien analysointi antoi viitteitä siitä, että vaikka diversiteettiä havaittiinkin runsaasti, otoskoko oli edelleen liian pieni ja johti lajimäärän aliarviointiin.

Avainsanat ¾ Nyckelord ¾ Keywords

Tomicus piniperda, pystynävertäjä, DNA analyysi*, Ophiostoma*

Muita tietoja ¾ Övriga uppgifter ¾ Further information

Ohjaajat prof. Frederick Asiegbu ja MMM Eeva Terhonen

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1. Introduction

1.1 Background

Insects and fungi share similarities in their ecological functions and structure since they contribute to nutrient cycling by obtaining nutrition from dead organic matter and regulate populations as parasites of plants, animals and members of their own kingdom. One structural similarity is the polymer 'chitin', that is part of the wall component of fungi as well as insects. These and other indications, such as the presence of mycangia in some bark beetles, suggest common or parallel evolution, towards an association (Wilding et al. 1989, Deacon 2006, Rohlfs 2005, Sauvard 2007).

Fungi have several important functions in the Boreal ecosystem, not only do they degrade dead wood but they also contribute to survival of trees and other plants by associating with their root as mycorrhizas. Many fungi can also act as pathogen of trees, or even as opportunistic saprotrophs on felled wood, and cause severe economic loss to the timber industry (Deacon 2006). Insects, such as some bark beetles, are known for their ability to attack and kill healthy trees; others in this group are merely secondary colonizers or attack weakened trees. It has been hypothesized that insects associate with aggressive fungi to defeat tree defenses. Aggressive beetles have been reported to associate with virulent fungi (Horntvedt et al. 1983, Jankowiak et al. 2009), however this might not always be the case (Six and Wingfield 2011). The associations between insects and virulent fungi might depend on insect population. Aggressive beetles are not always associated with virulent fungi, while non-aggressive beetles can associate with virulent fungi (Krokene and Solheim 1998, Viiri and Lieutier 2004). Soil conditions may affect the mycobiota associated with a specific insect. Soil texture, temperature, moisture, pH and other characteristics are determinant for the development of fungi and other microorganisms (Keller and Zimmermann 1989).

Competition and parasitism can also be types of interaction between these organisms. Many entomopathogenic fungi have been reported to associate with insects and some

have been employed as biological control agents (Humber and Hansen 2005, Wegensteiner 2007, Burjanazde 2010). Nevertheless, there is still a lack of necessary data required for their safe use, for example about their dynamics in different agroecosystems (Sun et al. 2008). In general, there are still many gaps to be filled in the knowledge of bark beetles and the associated fungi. Less attention has been paid to beetles that attack weakened trees and do not kill healthy trees, such as *Tomicus piniperda*, the pine shoot beetle.

The pine shoot beetle was once considered a dangerous pest in European pines and has been recently introduced to America. Studies have already focused on mycota associated with *Tomicus piniperda* in Europe (Bois et al. 1999, Solheim and Långström 1991, Jankowiak and Bilański 2007, Romón et al. 2007) and in eastern Finland (Linnakoski et al. 2010).

However, not much is known about the mycota associated with this insect in different geographical areas of Finland. Equally there is no information regarding epi- and endo-entomaphagus fungi associated with the insect. Such information could be interesting for further analysis on insect-fungus interactions.

1.2 Bark beetles

Bark beetles are insects from the order *Coleoptera* belonging to the *Curculionidae* family and *Scolytinae* subfamily. Bark beetle populations are very variable in space and time since they live in scattered habitats and new generations disperse searching for new breeding sites (Knížek and Beaver 2004). They are known for attacking several forest tree species and causing severe economic loss to forest plantations (Heritage and Moore 2001, Grégoire and Evans 2007). Damages due to pine bark beetles alone result in economic losses ranging from $100,000 to more than $25 million each year to landowners in Georgia, United States (Douce 1993).

Kirisits et al. (2007) reported that *Ips typographus* L. is the most destructive bark beetle of coniferous forests in Europe and there are records of it causing severe

losses in *Picea abies* Norway spruce forests. *Ips typographus* caused a reduction in production of 428,500 m^3 from 1991 until 1993 in north-eastern France. In the UK, half of the untreated young coniferous trees used for restocking plantations are lost due to the attack by large pine weevils, *Hylobius abietis*, over the first few years of establishment. *Hylobius abietis* is also a great threat to newly planted conifer seedlings after clearing in Scandinavia and large parts of central Europe (Heritage and Moore 2001, Viiri and Lieutier 2004, Petersson et al. 2006).

Several bark beetle species are known to exist in Finland, but most studies in Northern Europe have focused on the birch bark beetle and its fungal associates (Viiri 1997, Linnakoski et al. 2008, Linnakoski et al. 2009, Kamgan et al. 2010 cited from Linnakoski et al. 2010).

1.2.1 Pine shoot beetle

Tomicus piniperda is a bark beetle known as 'pine shoot beetle'. It is native to Europe, North west of Africa and North Asia but it has been introduced in North America, where it became a harmful pest (APHIS 2002). The pine shoot beetle is a polymorphic species with several haplotypes spread in Europe, whose Chinese and European populations have been isolated for around 0.6 MYA (Ritzerow et al. 2004).

It was formerly considered the most serious Scolytd pest of European pines and is still considered aggressive in Czech Republic, Poland, Slovakia and Spain (Thomas et al. 2004, Grégoire and Evans 2007). Presently, it is known only as a secondary colonizer. It mainly attacks *Pinus sylvestris* L., but can also attack other economically important forest tree species (Thomas et al. 2004). Due to the broad and continuous range of distribution of this main host and timber commercialization, *T. piniperda* populations have low genetical structure (Faccoli et al. 2005).

The pine shoot beetle is an opportunistic beetle that affects pines weakened by defoliation, either by total defoliation or if less than 10% of the treetop is present

(Annila et al. 1999). They also attack the shoots, dying or stressed pine trees, recently felled stumps and logs as well as bark mulch (APHIS 2002). However, it has been registered that in mass outbreaks, the beetle successfully attacks living trees (Kanchaveli and Supatashvili 1968 cited by Burjanazde 2010).

Figure 1: Adult *Tomicus piniperda* in collection sites

Figure 2: *T. piniperda* galleries in *Pinus sylvestris* felled logs

Adults of *T. piniperda* measure 3 to 5 mm in length (see Figure 1), are black and cylindrical and their elytral declivity is smooth and rounded. Females construct egg galleries in the phloem since they need fresh phloem for breeding, a typical bark beetle behaviour (Lanne et al. 1987). These are characteristically uniramous (see Figure 2) and range from 10 to 25 cm in length and about 2 mm in width. One egg is placed in each side of the gallery. After eclosion, the larvae construct 4-9 cm long galleries perpendicular to the egg galleries (Canadian Food Inspection Agency 2010) (see Appendix 2, Figure S1).

The inner bark, from the root collar to the middle or lower crown, serves as food for the larvae. The shoots become the overwinter sites for immature beetles, while short tunnels at the base of trees are the overwinter sites for adults (ibid, 2010). *T. piniperda* as well as other bark beetles are adapted to survive the winter by a series of actions such as accumulating supercooling substances. The supercooling point of *T. piniperda* reaches -18ºC thanks to the good insulation of its overwintering sites (Sauvard 2007). This beetle attacks pine shoots of any size, which eventually droop, become yellow and fall off in the months following winter. This may debilitate the tree to the point where *T. piniperda* can attack the trunk and lay its eggs in it (APHIS 2002).

The swarming period begins early in the spring and, although the swarming ends after warm days that reach above 10 to 14ºC, some reproductive activity will continue (Bakke 1968 in Saarenmaa 1989). The flight and swarming periods of *T. piniperda* depends on a temperature threshold. The flight initiation is inhibited at a temperature under 12ºC (Knížek and Beaver 2004). In the emergence flight of *T. piniperda* it can reach a distance of one kilometer from their brood trees, which is relevant for their dispersion (Sauvard et al. 1987). In Northern Finland the swarming period could be shorter than in the South, since a general feature of northern life is that of starting late and ending early (Saarenmaa 1989).

Although its main host is *Pinus sylvestris*, other species such as *Abies* and *Larix* can occasionally become hosts of this insect (Thomas et al. 2004). As other Scolytds, the

pine shoot beetle uses its host's volatiles such as α-pinene and ethanol to locate suitable breeding material (Schroeder and Lindelöw 1989). Tree vigour may alter the quantity or quality of volatiles released by the trees, which in turn may alter the trees' attractiveness to *T. piniperda* (Schroeder 1987).

These as well as other ecological characteristics are important when considering the sampling methods, especially if subjective sampling is to be considered. Subjective sampling was more effective than other methodologies to find specific bark beetle species according to Hedgren and Weslien (2008).

An attack of this bark beetle caused economic losses of 92 million Euro in Spain in 1989 and 4,500,000 hectares of trees were damaged by this insect in the 1990's (Grégoire and Evans 2007, Amezaga 1993 cited from Romón et al. 2007). Poland, Spain and Germany registered damages by this insect in large areas during the same decade while fewer hectares were affected in Portugal, Hungary, Slovakia, Romania and Switzerland (Grégoire and Evans 2007). According to Komonen and Kouki (2008), feeding activities of large populations of pine shoot beetle can cause economically significant growth loss. Therefore, bark beetles are a considerably important problem in forest management.

1.2.2 Fungi associated with bark beetles

Fungi and insects are equally indispensable for nutrient cycling and nutrient transport because they decompose organic matter (Rohlfs 2005). The fungal association with bark beetles are complex, sometimes strong and in others casual. In some cases insects can vector fungi but not all associations result in vectoring (Kirisits 2007). Some bark beetles have an invagination of cuticle known as mycangia, that carries fungal spores and mycelia. These mycangia are potentially a result of parallel evolution (Beaver 1989).

Fungi can also be carried phoretically on the surface of beetles, which is the case for those that lack mycangia such as *Tomicus piniperda* (Kirisits 2007). Fungi also serve

as food for the larvae, and vectored fungi could alter host plant availability and quality thus influencing the distribution and abundance of bark beetles (Scott et al. 2008, Masuya et al. 2009). However, a high population of larvae could compete with filamentous or saprotrophic fungi, altering local fungal population dynamics (Rohlfs 2005).

Ophiostomatoid fungi are a group of genera of Ascomycota such as *Ophiostoma*, *Ceratocystis, Grosmannia* and others that share morphological resemblance but differ phylogenetically (Wingfield et al 1993, Zipfel et al. 2006, Kirisitis 2007). These fungi can be identified for their long-necked perithecia, hyaline-mucilaginous ascospores and evanescent asci. The dispersal of many ophiostomatoid fungi depends on arthropods. It is believed that the aforementioned characteristics are connected with their dispersal biology (Masuya et al. 2009).

Some ophiostomatoid fungi are also known as blue-stain fungi that damage wood appearance and are detrimental for timber production (Wingfield et al. 1993, Deacon 2006). The genera *Ophiostoma* and *Ceratocystis* and its anamorphical species (*Pesotum*, *Leptographium*, *Hyalorhinocladiella* and *Sporothrix*) can cause sap-stain and tree-wilt diseases (Wingfield et al. 1993).

The association of ophiostomatoid fungi and bark beetles was considered either positive or neutral for the beetle. However evidence of antagonism has been found, where ophiostomatoid fungi presence decreases larval survival and competes with mutualistic fungal associates of beetles (Hofstetter et al. 2006, Lombardero et al. 2003). On the other hand, positive interaction can be observed with *Ips* beetles that can introduce blue stain fungi into egg galleries where larvae feed and will ultimately cause the death of the tree (Douce 1993).

Ophiostoma ulmi and *O. novo-ulmi* cause the Dutch elm disease and can use *Scolytus*, as well as *Hylurgopinus rufipes* as vector species. *Leptographium wingfieldii*, a pathogenic fungi, can associate with *Tomicus piniperda* in Sweden, Poland, Spain and even in North America after its introduction (Bois et al. 1999,

Jacobs et al. 2004, Hausner et al. 2005, Kirisits, 2007, Jankowiak and Bilański 2007, Romón et al. 2007).

The species *Hormonema dermatoides* (anamorph of *Sydowia polyspora*), *O. minus* and *L. wingfeldii* have been found to occur together when associated with *T. piniperda* in Sweden and France (Solheim and Långström 1991). The first two species have also been observed in association with *T. piniperda* in Germany (Grosmann 1931 cited by Solheim and Långström 1991).

Molds such as *Penicillium* and *Trichoderma* are commonly found associated with *T. piniperda*, and yeasts are not uncommon (Lieutier et al. 1989, Solheim and Långström 1991, Jankowiak 2006, Jankowiak and Bilański 2007, Linnakoski 2011a). Apart from *Ophiostoma minus*, another common associate with this bark beetle is *O. piceae*, in Spain and Poland (Romón et al. 2007, Jankowiak and Bilański 2007). *O. minus* has also been registered as associates of pine shoot beetle in eastern Finland and Russia (Linnakoski et al. 2010).

Other observed associates were *O. piliferum*, *Graphium* sp., *Leptographium procerum* (Jankowiak and Bilański 2007), *L. guttulatum*, *Ophiostoma ips*, *O. piceae*, *O. pluriannulatum*, *L. wingfeldii* (Romón et al. 2007) and *Beauveria bassiana* (Burjanazde 2010). In the aforementioned study in Poland (Jankowiak and Bilański 2007) it was found that ophiostomatoid fungi are not usually found in the body surface of *T. piniperda*. Nevertheless, *O minus* has been found to be carried phoretically or by mites in *Dendroctonus frontalis* (Lombardero et al. 2003).

Beauveria bassiana, an ubiquitous entomopathogen that can be used as biocontrol against insects (Ormond et al. 2010), is highly effective (71-100%) under appropriate climatic conditions against *T piniperda* (Lutyk and Swiezynska 1984). Isolates of *B. bassiana* from *T. piniperda* in Georgia were found to be highly effective - 81.5 to 100% - against these beetles and suggested as biocontrol for this and other bark beetle species (Burjanazde 2010).

Table 1: Fungi associated with *Tomicus piniperda* in Europe, Russia and North America

Fungi	Country or Region										
	S	F*	R	P	G	E	Fr	A	Sp	Gi	NA
Aurobasidium sp.							4				
Ambrosiella tingens	1,2,3										
Beauveria bassiana				15			14			18	
Ceratocystis minuta	3							10			
Cladosporium cladosporioides				15							
Cladosporium herbarum				16							
Diplodia pinea									17		
Graphium sp.				15,16		6, 8					
Graphium pycnocephalum				15							
Grosmannia cucullata			19								
Grosmannia piceiperda	7										
Hormonema dermatoides	7			16	20		4				
Leptographium chlamydiatum			19								
Leptographium guttulatum									17		
Leptographium lundbergii	1,3			15		6					13
Leptographium procerum				15,16		6					13
Leptographium wingfieldii	7			15,16	20	6, 8	4, 9	10	17		12
Mucor sp.				15			4				
Oidodendrum tenuissimum				16							
Ophiostoma brunneo-cilliatum			19				4				
Ophiostoma canum	1,2,3	19	19								
Ophiostoma clavatum	1,3										
Ophiostoma floccosum			19					11			
Ophiostoma huntii						6					
Ophiostoma ips	3						4		17		13
Ophiostoma minus	1,2,3,7	19	19	15,16	20		4, 5	10			13
Ophiostoma olivaceum											
Ophiostoma picea	1,3,7			15,16		6			17		

Table 1 continued

Fungi	Country or Region										
	S	F*	R	P	G	E	Fr	A	Sp	Gi	NA
Ophiostoma piceaperdum	7							10			
Ophiostoma pini											
Ophiostoma piliferum	1,2,3,7			15		6					
Ophiostoma pluriannulatum									17		
Oidiodendron tenuissimum				16							
Penicillium spp.				16			4				
Phialophora clavispora				16							
Trichoderma spp.				15			4				

N: Norway, S: Sweden F: Finland; R: Russia; P: Poland; G: Germany; E: England; Fr: France; A: Austria; Sp: Spain; Gi: Georgia; NA: North America

* Eastern Finland

Table prepared by the author based on the following authors:

1: Mathiesen (1950), 2: Rennerfelt (1950), 3: Mathiesen-Käärik (1953), 4: Lieutier et al. (1989),5: Piou et al. (1989), 6: Gibbs and Inman (1991), 7: Solheim and Langstrom (1991), 8: Wingfield and Gibbs (1991), 9: Bois et al. (1999), 10: Kirisits et al. (2000), 11: Lin (2003), 12: Jacobs et al. (2004), 13: Hausner et al. (2005), 14: Humber and Hansen 2005(6), 15: Jankowiak (2006), 16: Jankowiak and Bilański (2007), 17: Romón et al. (2007), 18: Burjanazde (2010), 19: Linnakoski (2011), 20: Grosmann (1931) cited by Solheim and Langstrom (1991)

Many of the aforementioned ophiostomatoid fungi were also isolated from galleries of trap logs, standing trees and directly from the *T. piniperda* in Canada (Hausner et al. 2005). However, the association of ophiostomatoid fungi with the pine shoot beetle has been registered as loose (Kirisits 2007). A summary of some registered cases of fungi associated with *T. piniperda* in Europe and North America is shown in Table 1.

Most fungi associate randomly with beetles. The most common associates are less diverse and occur in a range of 2 to 3 fungal species per bark beetle (Six and Wingfield 2011). Among the common associates usually two of them are virulent while the rest is less virulent (Kirisits 2007). Many fungal species associated with *T. piniperda* have been found to associate with other scolytids that also target pine or even other conifers (Solheim and Långström 1991, Jankowiak et al. 2009).

1.2.3 Relevance of the relationship between bark beetles and fungi

A major concern regarding bark beetles vectoring forest pathogenic fungi is the problem of alien diseases entering native forests. Humble and Allen (2006) mentioned that insects such as the brown spruce longhorn beetle, *Tetropium fuscum*, can vector native or alien fungal pests as well as nematodes or acari.

However, there is not enough knowledge on how many fungi are being introduced by insects and how many native fungi can have a new spread path through alien insects. Furthermore, knowledge of the association between mites, nematodes and fungi that are vectored by these beetles is still obscure (ibid 2006).

Although few species are aggressive enough to overcome plant defences, some bark beetles are able to successfully colonize and kill healthy or damaged trees. Nevertheless, it has been hypothesized that they can receive help from fungi to overcome tree defences (Krokene and Solheim 1998 , Urbanek 2009). Consequently, economic losses can be more relevant for forest plantations when considering the joint forces of these two potentially damaging organisms.

Additional concern arises when climate change risks are considered. Climate Change predictions indicate that Scandinavian countries will in future experience a warmer climate. Environmental disturbances usually give rise to outbreaks of forest pests resulting in weakened host trees (Faccoli 2009). According to Jönsson and Bärring (2011), an increase in temperature of 2º or 4ºC could induce a shift from one to two generations of *Ips typographus* per year in South Scandinavia. The reason behind this is that a warmer climate accelerates the initiation of the first generation, and accelerates the transition from egg to mature beetle, thus increasing the probability of an extra generation per year. A similar situation could occur with other bark beetles.

However, in the case of the pine shoot beetle other factors besides temperature seem to inhibit the occurrence of more than one generation per year. The photoperiod or

the need for low temperatures to achieve maturation have been suggested as potential inhibitors, but more studies are needed on the subject (Sauvard 2007).

Nevertheless, Lindner et al. (2008) suggest that *T. piniperda* will likely benefit by the weakened trees affected by the outbreak of other pests in Fennoscandic forests.

The aforementioned ideas reflect the traditional view of a mutualistic association between fungi and insects in relation to their pathogenicity. Nevertheless, virulent fungi do not always associate with tree-killing bark beetles. Pathogenicity could potentially achieve a role for the fungi rather than a favour for the beetle (Six and Wingfield 2011). Also contradicting the traditional view, is the fact that non aggressive bark beetles have been found to be able to associate with virulent fungi, and that non-pathogenic fungi can also be associated with aggressive bark beetles (Krokene and Solheim 1998).

Due to the aforementioned reasons, better understanding of the association between beetles and fungi will help when developing forest management decisions.

1.2.3.1 Physiochemical requirements of fungi associated with insects

Masuya et al. (2009) suggested that physiological characteristics of fungal species cause the breeding habitats of the beetles to limit the frequency of occurrence of fungal population. Highly virulent pathogenic fungi may have a role in the establishment mechanisms of bark beetles in the tree host when the association is constant (Lieutier et al. 1989). However, as mentioned before, virulence in fungi could have another role than collaboration with associated bark beetle (Six and Wingfield 2011).

Temperature plays a key role in determining the relative abundance of bark beetle-vectored fungi (Rice et al. 2008 and Six et al. 2005 cited by Persson et al. 2009). Viiri and Lieutier (2004) indicated that the roles of associated fungi may differ under different environmental conditions.

The mycota associated with beetles depends on environmental restrictions. Environmental restrictions can decrease the beetle population (Deacon 2006). Viiri and Lieutier (2004) indicated that pathogenic fungi associated with beetles could be replaced by other species during endemic periods. For example, the frequency of *Ceratocystis polonica* is very low during periods of low population of *Ips typographus* but high in the epidemic phase when they attack trees.

1.2.3.2 Physiochemical requirements of the insect host

As with any other diseases or pathogens, a susceptible host is needed for an insect to infect it. A stressed tree can become a target for insects, and drought is one of the main stress factors inducing bark beetle infestations. Management practices that allow higher density and older trees to grow together, thus causing them to compete for water, could create a suitable environment for beetle infestation (Faccoli 2009).

Bark beetles can attack living trees and this risk is related to exposure, age, nutrients, water supply, density of beetles and weather conditions (Urbanek 2009). Different beetles have different temperature, sunshine or altitude needs for their feeding activities and larval development (Petersson et al. 2006, Wainhouse and Brough 2007).

Faccoli (2009) found that an increase in temperature in springtime does not affect the development timing of *Ips typographus*. Furthermore, spring droughts increase damages caused by *I. typographus* in the following year, while a warmer spring affects this insect's phenology. The development of a third generation depends on the summer photoperiod rather than temperature.

Consequently, certain environmental conditions that favour the reproduction and outbreak of pest insects ought to be avoided when planning forest management. These conditions should be well known before hand which merits the need for further research.

1.3 Aims of the study

The specific aims of the study are to:

1. Identify fungal diversity potentially associated with *Tomicus piniperda*, in Northern and Southern Finland.
2. Evaluate shifts in the populations of fungi communities as a result of the change in environment, not only latitudinal but also regarding its location on the interior or exterior of the insect.

Hypothesis:

1. *Tomicus piniperda* is associated with different pathogenic and non pathogenic fungi in Northern and Southern Finland
2. There is a shift in the fungal community associated with *Tomicus piniperda* with changing latitude and habitat

2. Materials and Methods

2.1 Study area

The sampling method for this experiment was subjective sampling, considering that *Tomicus piniperda* affects weakened pine trees. Therefore, the targeted samples are those where weakened pines have been suffering stress by fire, pollution or fungi attack or that have been recently felled (see Appendix 2, Figure S2). Since the intention was to compare Northern and Southern Finland, the two different geographical areas were designated as North and South (see Appendix 2, Figure S3).

Tomicus piniperda samples were collected in Northern Finland on May 19th, 2010 (see Appendix 2, Figure S4). The samples consisted of approximately 38 insects per site from 23 sites around Rovaniemi Railway Station (66°29′N, 25°42′E, 86 m above sea level). The insects were collected from fresh logs of *Pinus sylvestris*. The largest city near the station is Rovaniemi, the capital of Lapland where the mean temperature is 0.2 ºC and mean annual precipitation is 550-600 mm (World Weather Information Service 2011). The sites are located in the transition zone of of the northern and middle boreal vegetation zone where most mineral forests are mixed. They are either dominated by Norway spruce, Scots pine or both, and mixed with birches. Most sites are either moist or dryish in their fertility (R. Jalkanen, personal communication, April 1, 2011).

The sampling site at Hyytiälä Experimental Station (61°51´N, 24°17´E, 181 m above sea level) is situated in Southern Finland and the forest around the station is representative of the boreal coniferous type (see Appendix 2, Figure S5). The forest is dominated by 40-year old Scots pines (*Pinus sylvestris* L.) for about 200 m in all directions, extending to the north for about 1.2 km. The largest city near the station is Tampere, and the terrain is subject to modest height variation. The annual mean temperature is 3ºC and mean precipitation is 700 mm (Hari and Kulmala 2005).

Samples were collected from seven different sites in Hyytiälä on June 3rd and 6th, 2010. The samples were collected from recently felled logs and trees affected by storms. About 28 insects were collected per site in Hyytiälä.

2.2 Collection and fungal isolation

The insect were collected manually using forceps and placing them in microcentrifuge tubes of 1.5 mL. The samples were stored at -5ºC for two weeks to achieve the senescence of the insects.

The insect samples were divided in equal amounts into North and South. On average, five insects were placed per Petri dish. Half of the insects from every site were analyzed for epi-mycota and the other half for endo-mycota. The medium used for the isolation was autoclaved malt extract agar (1.5% Bacto™ malt extract and 1.0% Difco Bacto™ agar). The insects used for isolation of epi-mycota were neither sterilized nor crushed. They were simply placed entirely in Petri dishes containing agar. For the isolation of endo-mycota, the insects were surface sterilized with 70% ethanol for 30s, then crushed with mortar, and placed in Petri dishes containing ME agar.

After the placement, the petri dishes were incubated at room temperature for two weeks. The petri dishes containing insects to be studied for endo-mycota were left in a box under the table, in order to receive less light and provide an environment more suitable for that kind of fungi.

To indicate whether the Petri dish contained insects from the North or South, and endo- or epi-mycota, the following symbols were used:

S: South
N: North
I: endo-mycota (insects sterilized and crushed)
E: epi-mycota

Monitoring of samples consisted of daily observations looking for mycelium growth. Whenever mycelium growth was observed, attempts were made to subculture the fungi. The fungi were isolated under sterile hood with autoclaved forcerps to avoid contamination.

Several isolation attempts took place over a period of three months, until a pure culture was obtained. Isolation consisted of transferring a piece of newly emerging hyphae to another petri dish using a sterile needle. The purpose was to isolate a pure culture in each petri dish until all fungal species from the collected insects were isolated. The needle was submerged in 70% ethanol and flamed before and after each isolation. A new culture medium (50 mg/mL Ampicillin, 1% Difco™ potato dextrose broth, 2% Bacto™ peptone and 1.5% Bacto™ agar) was made specially for the yeasts, containing an antibiotic to avoid bacteria from growing in the Petri dishes.

Thereafter, the cultures were kept in the incubator at 28ºC for one to two weeks until the yeast had grown. If the culture was not pure, it was discarded, to be remade until a pure culture was obtained. Fungal morphotypes were grouped with the aid of microscope. The cellophane membranes were autoclaved twice before placing them over the agar plates. Pure cultures of representatives of each morphotype from the different latitudes and places were then transferred to the membrane on agar plates.

2.3 DNA extraction

DNA was extracted using a standard cetyltrimethylammonium bromide (CTAB) method routinely used in the laboratory for isolating genomic DNA from *Heterobasidium annosum*.

For each sample, a portion of fresh mycelium was extracted and transfered to a 1.5 ml eppendorf tube. Afterward, samples were ground and homogenized with sterilized sand and 100 µl of 2% CTAB using a micropestle (see Appendix 1 for details of preparation). The sample was incubated for an hour at 65ºC. Subsequently, the sample was treated with equal amounts of chloroform:isoamyl alcohol (24:1), and

stirred manually for 7 minutes before being centrifuged at 13000 rpm for 15 minutes. New eppendorf tubes were used to collect the upper aqueous phase after centrifugation This procedure was repeated twice. The resulting aqueous phase was transferred to a new eppendorf tube and two volumes of cold isopropanol were added. This preparation was then left on ice for 30 minutes. After that, the samples were centrifuged at 13000 rpm for 20 minutes. The resulting supernatant was removed carefully and the pellet was washed with 200 µL of cold isopropanol. The samples was then centrifuged at 7500 rpm for 5 minutes. The supernatant was again removed and the pellet was left to dry under laminar flow. Finally, the pellets were resuspended in 40 µL of TE buffer and stored at -20ºC.

2.4 Polymerase chain reaction (PCR)

2.4.1 DNA purity assessment

It is necessary to asses the purity and concentration of DNA before proceeding with the PCR. For that purpose a spectrophotometer, NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA) , was used. The assessment required using 1.5 µl of each sample. In the case of those whose concentration and quality was very low, the previous steps (see section 2.3) were repeated until obtaining a good quality sample. In total, there were 43 groups of potentially different species and 87 DNA samples were extracted. They were kept in a box at -20ºC. After assessing the DNA concentration of those samples it was possible to determine the dilution needed for the PCR.

2.4.2 PCR protocol

Based on the NanoDrop results, the necessary quantity of each sample was transferred to new eppendorf tubes and diluted with autoclaved MQ water in order to obtain a final volume of 10 ng/µL of DNA to be used for amplification. In the case of samples with low DNA concentration, 10 µL of each of those samples was mixed with 40 µL autoclaved MQ water. Primers ITS1 and ITS4 were used to amplify the

ITS regions. Gene reactions were amplified in a final volume of 25µL placed in 200 µL microcentrifuges, containing 5 µL of diluted DNA and 20 µL of reaction mixture (see Annex for reagents). The PCR protocol consisted in the initial denaturation of the DNA at a temperature of 94º C for 4 minutes. The thermocycler used for DNA amplification was set in the following program: initial denaturation at 94ºC for 4 minutes; 30 cycles of denaturation at 94ºC during 45 seconds, annealing for 45 seconds at 55ºC and extension for 10 minutes at 72ºC; final extension at 72ºC for 10 min. The final products were kept at -20ºC.

2.4.3 Gel electrophoresis of DNA

In order to assess the DNA amplification as well as its purity, the PCR products were run on agarose gel. The samples were kept on ice and 5 µL were mixed with 1.5 µL loading buffer (see Annex for solutions). The samples were then run in agarose gel stained with ethidium bromide (120 V for 25 minutes). Afterwards the picture of the gel was taken with gel image system (Molecular Imager Gel Doc XR+ System).

After obtaining positive PCR results the samples were sent to be sequenced at the Haartman Institute, in Helsinki, Finland. ITS sequences were blasted against sequences from the Genbank database to establish the fungal taxa (see further details below).

2.5 Statistical and Phylogenetic analysis

After obtaining the sequences of 53 of the fungal isolates, the chromatograms were manually analysed using Flinch TV (http://www.geospiza.com/Products/finchtv.shtml), a chromatogram viewer that can display an entire trace in a scalable multi-pane.

Following this step they were further refined using Fungal ITS extractor (http://www.emerencia.org/FungalITSextractor.html), which is free open source software used to extract ITS1 and ITS2 subregion from the sequences. Then, the

sequences were blasted against GeneBank/NCBI sequences. In order to determine species, taxon or order, the closest BLAST matches and morphological description were used. Additionally, to obtain further confirmation, phylogenetical analysis of the sequences using ClustalX (Larkin et al. 2007) and MEGA5 (Tamura et al. 2011) were used to draw a phylogenetic tree. Sequences were aligned using multiple sequence alignment and analysed with maximum parsimony and maximum likelihood using Clustal X. Additionally, pictures of the cultures were taken with a microscope camera and the software getIT.

The fungal population of the North, South, the endo- and epi-mycota of the respective areas were tested with a mixed model using IBM SPSS Statistics 19.0. In the model, sites were used as random subject and insect as repeated subject.

Species richness and diversity indexes were calculated with the programme Estimates 7.5 (Colwel 2009), a software application that measures several biodiversity indexes.

It should be noted that there were more sites and insects collected from the North than from the South, consequently in the statistical analysis only 7 sites from the North were considered against the 7 sites from the South.

3. Results

3.1 Overview of the fungal population

From the 1088 insects collected, both from South and North, a total of 3073 fungal isolates were obtained. These fungal isolates where assigned to 26 different taxonomical units. From these, 23 were identified to the level of family, genus and even species, as observed in Table 2. There where 3 unidentified morphotypes that where considered as different taxonomic units.

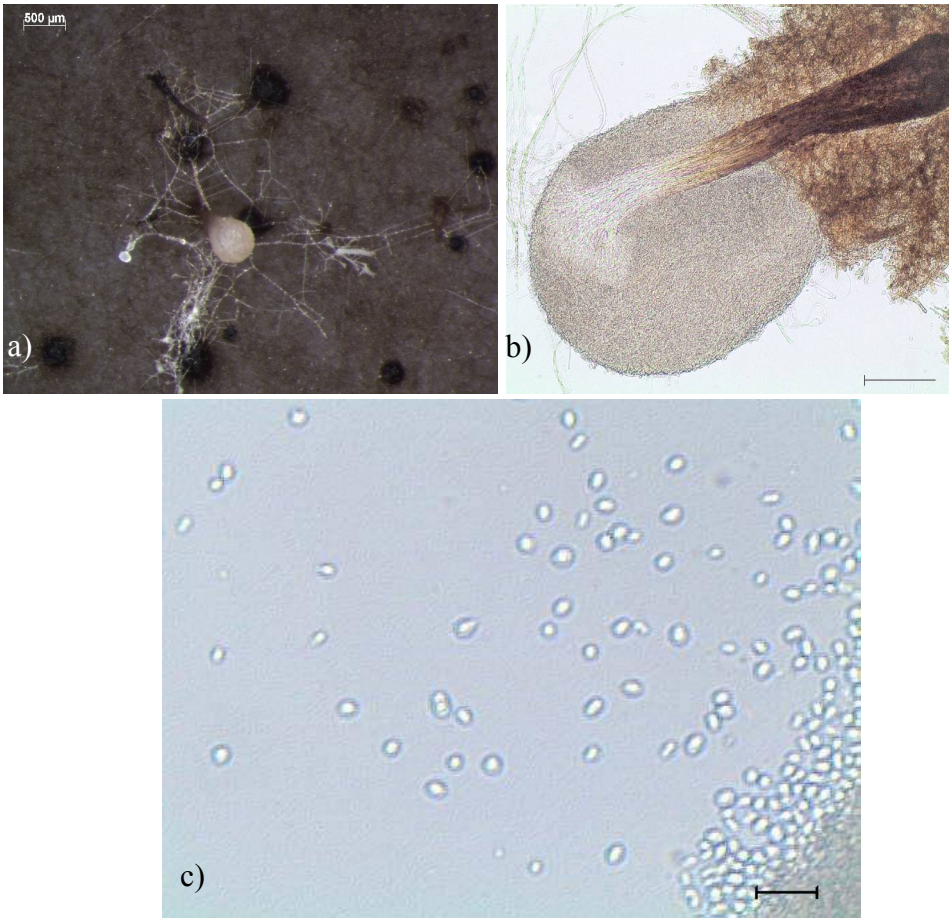

Figure 3: a) and b) represent *Pesotum*-anamorphs typical to many *Ophiostoma* spp.; c) spores of *Ophiostoma canum*. Scale bars: a) 500 µm; b) 40 µm; c) 10 µm.

Another observation was that the majority of the identified isolates belonged to only two phyla: Ascomycota and Zygomycota. The Ascomycotas in the study belonged to

four different classes: Dothideomycetes, Euromycetes, Saccharomycetes and Sordariomycetes.

The Sordariomycetes, with 7 taxa, were the most commonly isolated in all cases, followed by the Euromycetes and the Saccharomycetes. Euromycetes were represented by 6 taxa, Saccharomycetes by 5 taxa and Dothideomycetes by 2 taxa. The identified Zygomycetes were represented by 2 taxa. To assign the level of species of *Ophiostoma canum* (see Figure. 3), the morphology was taken into consideration.

The most abundantly isolated fungi belonged to the phylum Ascomycota. The most represented class was Sordariomycetes, that reached 51.17% of the Ascomycotas, followed by Euromycetes at 26.4% of all the isolations from that phylum. Saccharomycetes represented 21.91% of the Ascomycetes and Dothidiomycetes were only 0.52%.

3.2 The effects of site on the mycobiota population

Nine species from the ones isolated from the southern insects were common to all southern sites: *Penicilium* sp, *P. brevicompactum*, *P. velutinum, Ophiostoma minus, Ophiostoma canum*, *Trichoderma* sp, *Mortierella* sp., *Umbelopsis* sp. and one unidentified species. Among the isolates from the southern insects, there were 18 identified species and 3 unidentified species.

There were representatives from 20 species isolated from the 7 sites studied in the North. Five species isolated from the northern insects were common to all studied sites: *Penicilium* sp, *Kurushia* sp., *Beauveria bassiana, Ophiostoma canum* and *Mortierella* sp.

Eight fungal species were found to be common to both the southern and the northern insects: *Penicilium* sp, *Penicillium velutinum*, *Kurushia* sp*., Ophiostoma canum, Trichoderma* sp., *Trichoderma viride*, *Mortierella* sp. and *Umbelopsis* sp.

Table 2: Fungi isolates from *T. piniperda*

Isolate	GenBank accession no. of best matches	Mi(%) / Qc(%)	Description of best matches	Suggested isolate name	Site	Freq.	Phylum
N22	EF126738	91/100	*Geotrichum restrictum*	*Ascomycete sp*	North 23	1	A
S27	EF126738	88/65	*Geotrichum restrictum*	*Dipodascaceae sp*	South 1	32	A
N35	FJ903282	99/90	*Grosmannia olivacea*	*Grosmannia sp*	North 14	14	A
S5	AM943890	91/99	*Ophiostoma minus*	*Ophiostoma minus*	South 2	35	A
S1	HQ115646	97/100	*Arthrinium sacchari*	*Arthrinium sacchari*	South 7	30	A
N23	GU566276	99/100	*Beauveria bassiana*	*Beauveria bassiana*	North 23	718	A
S55	GU062258	96/96	*Umbelopsis isabellina*	*Umbelopsis sp*	South 7	98	Z
S50	FJ872076	95/98	*Umbelopsis sp*	*Umbelopsis sp*	South 5	98	Z
N25	FJ872073	99/100	*Trichoderma viride*	*Trichoderma viride*	North 4	217	A
S14	GU934567	99/100	*Trichoderma viride*	*Trichoderma viride*	South 5	217	A
S47	FJ872073	89/100	*Trichoderma viride*	*Trichoderma sp.*	South 2	128	A
S18	FM200701	100/72	*Fungal endophyte*	*Unidentified*	South 2	69	
S48	GU237907	94/74	*Phoma sylvatica*	*Ascomycete sp*	South 4	1	A
S7	AJ878778	99/100	*Mortierella humilis*	*Mortierella sp.*	South 5	160	Z
S34	AF033448	93/100	*Penicillium velutinum*	*Penicillium sp.*	South 5	383	A
S58	EU541359	95/99	*Candida oleophila*	*Candida sp. 1*	South 1	19	A
N13	EU484318	97/96	*Candida fructus*	*Candida sp. 2*	North 5	3	A
S57	AY761156	98/97	*Saccharomycetaceae*	*Saccharomycetaceae sp*	South 1	16	A
S2	HM031489	94/98	*Ophiostoma canum*	*Ophiostoma canum*	South 2	258	A
S15	GU237907	99/99	*Phoma sylvatica*	*Phoma sp*	South 1	4	A
S19	AJ876492	91/100	*Umbelopsis ramanniana*	*Umbelopsis sp*	South 2	98	Z
N31	EF568065	99/99	*Kuraishia capsulata*	*Kuraishia sp*	North 15	529	A
S49	EU877261	98/100	*Sydowia polyspora*	*Sydowia polyspora*	South 4	10	A
N1	GU566252	100/99	*Penicillium spinulosum*	*Penicillium sp.*	North 17	383	A
S62	AY373927	99/100	*Penicillium raistrickii*	*Penicillium sp.*	South 1	383	A
S25	AF033410	100/100	*Penicillium spinulosum*	*Penicillium spinulosum*	South 1	24	A
S46	GU566252	98/100	*Penicillium spinulosum*	*Penicillium sp.*	South 2	383	A
N4	GU566252	99/100	*Penicillium spinulosum*	*Penicillium spinulosum*	North 17	24	A
S22	GU566252	99/100	*Penicillium spinulosum*	*Penicillium spinulosum*	South 5	24	A
S10	AB479306	99/100	*Penicillium brevicompactum*	*Penicillium brevicompactum*	South 2	127	A
S32	AJ878778	98/100	*Mortierella humilis*	*Mortierella sp.*	South 5	160	Z
N32	AY373927	99/100	*Penicillium raistrickii*	*Penicillium sp.*	North 10	383	A
S26	AY373927	98/100	*Penicillium raistrickii*	*Penicillium sp.*	South 1	383	A
N12	AY373927	100/100	*Penicillium raistrickii*	*Penicillium sp.*	North 5	383	A
N28	AY373927	99/100	*Penicillium raistrickii*	*Penicillium raistrickii*	North 10	**7**	A
S45	AF033448	96/99	*Penicillium velutinum*	*Penicillium sp.*	South 2	383	A
N16	AY373927	100/100	*Penicillium raistrickii*	*Penicillium sp.*	North 5	383	A
N14	AF033448	100/100	*Penicillium velutinum*	*Penicillium velutinum*	North 19	145	A
N30	AF033448	100/100	*Penicillium velutinum*	*Penicillium sp.*	North 19	383	A
S21	AF033448	100/100	*Penicillium velutinum*	*Penicillium velutinum*	South 3	145	A
N10	GU566224	99/100	*Penicillium griseofulvum*	*Penicillium griseofulvum*	North 5	35	A
N27	AM901674	100/100	*Penicillium sp.*	*Penicillium sp.*	North 5	383	A

A: Ascomycetes; Z: Zygomycetes

3.3 Geographical location and its effect on fungal population

Beauveria bassiana was isolated mainly from the North, both as epi and endo-mycota and was the second most common isolate - 15.10% - from the study (see Figure 4).

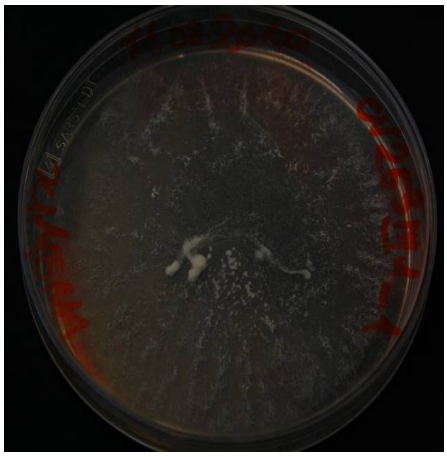

Figure 4: *Beauveria bassiana*, sample from the North.

The remaining taxa, with the exception of the molds, represent less than 5-10% of the isolations. Nine taxa were exclusively isolated from the southern insects while 12 were common to both latitudes (see Figure 5).

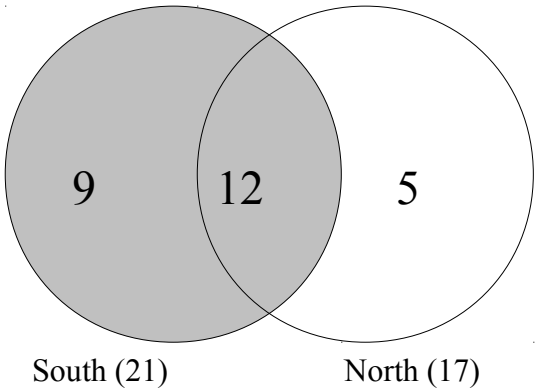

Figure 5: Number of common and exclusive fungi from North and South

There where 2 *Candida* sp. isolated in the study that were considered as different species according to the blast results and the phylogenetical analysis. One of these was isolated exclusively from the North and the other only from the South. Five

species were exclusively isolated from the North: *Penicilium griseofulvum*, *P. raistrickii*, *Beauveria bassiana*, *Grosmannia* sp. and one of the *Candida* sp.

Almost all taxa were found in the North and South, epi- and endo-mycota, which could indicate low specificity to the area. There where 2 taxa exclusively from the North epi-mycota group, *Penicillium raistrickii* and *Grosmannia* sp., and 2 exclusive to North endo-mycota group.

Four identified and 2 unidentified isolates were found exclusively in the South: *Phoma* sp., *Penicillium brevicompactum*, a Saccharomycete and the other Candida sp.

3.4 Epi and endo- mycota

Only one taxon was exclusive from the South epi-mycota and 3 taxa were only isolated as endo-mycota, as seen in Figure. 6 a). Sixteen species from the ones isolated in the southern insects were common to both the epi- and endo-mycota population.

Eight species were isolated from all 7 sites from the surface of insects, which represents 53.02% of the total isolated species from the epi-mycota. These species were *Penicilium* sp, *P. brevicompactum*, *Ophiostoma canum*, *Trichoderma* sp, *Mortierella* sp., *Umbelopsis* sp. and 2 unidentified species. There were no common endo-mycota isolates from all sites in the South.

The epi-mycota isolates common to the studied sites of the North were *Ophiostoma canum* and *Mortierella* sp, which represent 23.16% of the number of species isolated in the surface of the northern insects. The number of shared isolates in the epi and endo-mycota in the northern insects is summarized in Figure 6 b).

Six taxa of epi-mycota present in the southern insects were not found in the surface of northern insects. Twice as many taxa were common in all the epi-mycota

population. Eight taxa were common in the endo-mycota, while 11 from the ones found in the southern insects were not present in the northern insects (see Figure 6).

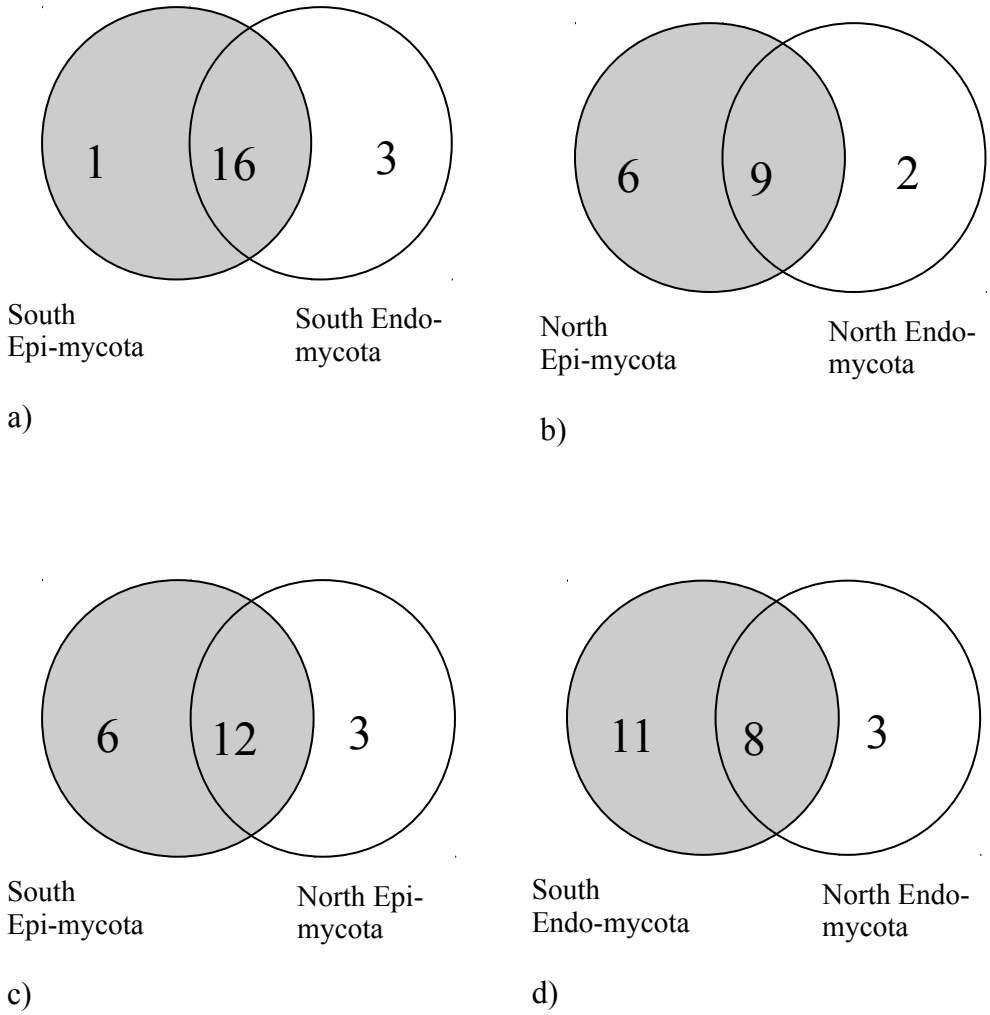

Figure 6 a) Number of common and exclusive fungi from South epi- and endo-mycota. b) Number of common and exclusive fungi from North epi- and endo-mycota. c) Number of common and exclusive epi-mycota from South and North. d) Number of common and exclusive endo-mycota from South and North

Eight taxa were isolated from all, epi- and endo-mycota from North and South: *Kuraishia* sp., *Penicillium* sp., *Ophiostoma canum*, *Trichoderma viride*, *Mortierella* sp., *Penicillium velutinum*, *Trichoderma* sp., and *Umbelopsis sp.* Altogether, the aforementioned taxa represent 62.54% of all the studied isolations.

Two of the fungal species isolated exclusively in the North were at the same time primarily observed from the surface of the insects: *Penicillium raistrickii* and *Grosmannia* sp. Isolates that were exclusive from the north epi-mycota were *P. griseofulvum* and *Candida* sp.

3.5 Diversity indexes

The South was undersampled according to the diversity indices, as seen in Table 3. This assumption was supported by the high difference between the data observed in Chao 1 and Chao 2 in contrast with the species richness, specially in the case of the South. They also reflect how many species were missed, by considering those that were found only once (singletons) or twice.

Table 3: Diversity indexes according to geographical location and epi- or endo-mycota.

Latitude	Fungi	Species richness	Abundance	Fisher's α	Shannon's	Chao 1	Chao 2
North	Epi-mycota	15	353	3.25	2.16	15.34	101.08
North	Endo-mycota	11	366	3.96	2.47	20.7	23.88
South	Epi-mycota	18	402	4.28	2.58	23.8	28.33
South	Endo-mycota	19	354	4.48	2.62	26	30.5

However, Shannon's diversity index ranges from 1.5 to 3.5, where the upper limit indicates high diversity. In the present study, the index ranged from 2.16 to 2.62, which suggests there was a relatively high diversity of species in the studied sites. The highest diversity was observed in the South and in the endo-mycota group, and the lowest was observed in the epi-mycota group of fungi in the North. The same results are confirmed by the Fisher's alpha index, which indicates that the Southern fungal population as the most diverse and rich when compared to the one in the North.

Even when considering all sites in the North, there were on average 2.84 different fungal taxa per insect in North epi- and 2.37 in North endo-mycota. While in the case of the South there were 4.10 fungal taxa per insect in the South epi and 3.47 fungal

taxa in those considered South endo-mycota. This suggest that a higher amount of different species of fungi were found in *Tomicus piniperda* in Southern Finland.

3.6 Similarity

The similarity indexes obtained from Estimates are displayed in Table 4. The Jaccard, Sorensen, Morisita-Horn and Bray-Curtis indexes were higher for South epi and endo-mycota.

Table 4: Similarity indexes of mycota isolated from *Tomicus piniperda* in different geographical locations and insect position.

Comparison		Shared Species	Jaccard Classic	Sorensen Classic	Morisita-Horn	Bray-Curtis
North Epi-mycota	North Endo-mycota	9	0.529	0.621	0.659	0.509
North Epi-mycota	South Epi-mycota	12	0.571	0.824	0.602	0.527
North Epi-mycota	South Endo-mycota	12	0.545	0.8	0.448	0.379
North Endo-mycota	South Epi-mycota	8	0.381	0.621	0.286	0.352
North Endo-mycota	South Endo-mycota	8	0.364	0.533	0.201	0.294
South Epi-mycota	South Endo-mycota	16	0.762	0.914	0.873	0.688

This indicates that the fungi isolated from the South are the ones with the highest similarity with the highest number of shared species. Similar observation was made when comparing North epi-mycota with both South epi and endo-mycota.

Table 5: Test of Fixed effects regarding frequencies in the mycota isolated from *T. piniperda* in different geographic locations

Source	Numerator df	Denominator df	F	Sig.
Intercept	1	79.172	1102.134	0.000
Latitude	1	79.174	43.569	0.000
Endo/ Epi-mycota	1	78.406	1.312	0.256

a. Dependent Variable: Frequency.

Table 6: Pairwise comparison of mycota isolated from *T. piniperda* collected at different geographic locations

Latitude	Latitude	Mean Difference	Std. Error	df	Sig.a	Lower Bound	Upper Bound
South	North	1.267*	0.192	79.174	0.000	0.885	1.649
North	South	-1.267*	0.192	79.174	0.000	-1.649	-0.885

Table 7: Pairwise comparison of epi vs endo-mycota isolated from *T. piniperda*

Endo/ Epi-mycota	Endo/ Epi-mycota	Mean Difference	Std. Error	df	Sig.a	Lower Bound	Upper Bound
Epi-mycota	Endo-mycota	0.217	0.19	78.406	0.256	-0.16	0.595
Endo-mycota	Epi-mycota	-0.217	0.19	78.406	0.256	-0.595	0.16

Table 8: Mann-Whitney and Wilcoxon tests for epi- and endo-mycota isolated from *T. piniperda*

Test	Frequency
Mann-Whitney U	26380.000
Wilcoxon W	55541.000
Z	-1.651
Asymp. Sig. (2-tailed)	0.099

Table 9: Mann-Whitney and Wilcoxon tests for Northern and Southern mycobiota isolated from *T. piniperda*

Tests	Frequency
Mann-Whitney U	13120.000
Wilcoxon W	52741.000
Z	-10.275
Asymp. Sig. (2-tailed)	0.000

The Sorensen index indicates that, after the South epi- and endo-mycota fungal populations, the mycota isolated from the North epi-mycota group were similar to the ones isolated from the South epi-mycota group. The other indexes also indicate that this is the second group with highest similarities.

The least similar were the North endo-mycota and the South endo-mycota. The results from the mixed model indicate that there is a significant difference between the fungal populations in the North and in the South as seen in Tables 5 - 6 and 9 (see Appendix 2, Figure S6). On the other hand, it becomes clear when observing Table 5, 7 and 9 that differences between the epi- and endo-mycota are negligible (see Appendix 2, Figure S7). The Mann-Whitney test in Table 8 indicates that there is a 95% chance that no significant difference between the median of the epi- and endo-mycota exists. On the other hand, the results of the Mann-Whitney test shown in Table 9, that compares medians from mycota isolated from the Northern and Southern insects, indicate that they are siginificantly different at the same confidence level.

3.7 Ascomycetes

3.7.1 Sordariomycetes

The most common isolate was *Beauveria bassiana,* a Sordariomycete, that was only isolated from the North. The most common Sordariomycete isolated from both latitudes and insect parts was *Ophiostoma canum* (see Appendix 2, Figures S8-S9).

Other important isolates from this group were *O. minus* and *Grosmannia* sp. *O. minus* was found mostly in the South while *Grosmannia* sp. was isolated only from the surface of the insects from the North. *Ophiostoma minus* represented only 2.18% of the studied isolates and *O. canum* only 7.97% of the total isolates.

Sydowia polyspora, which is associated with current season needle necrosis on *Abies spp* (Talgø et al. 2010), was also isolated but only as 0.54% of the isolates.

3.7.2 Saccharomycetes

The Saccharomycetes represented 14.76% of the studied isolations and from this group *Kuraishia* sp. alone represents 62.04% of the yeast isolated in the study (see Appendix 2, Figure S10).

3.7.3 Euromycetes

The Euromycetes represented 35.47% of all the studied isolations, and in all cases these belonged to the genus *Penicillium*. These are common saprotrophs, cosmopolitan genus that are present in the soil and that can grow on decayed wood (Deacon 2006). Other common saprotrophs isolated in the study belonged to the genus *Trichoderma*, that represented 8.41% of the isolations.

3.8 Zygomycetes

Only two Zygomycetes belonging to the sub-phylum Mucoromycotina (Stajich et al. 2009) where identified to the level of genus, the mitosporic fungi *Mortierella* and *Umbelopsis*. *Mortierella* can degrade chitin such as the exoskeleton of insects (Deacon 2006), and was isolated in both latitudes and both insect parts. Meyer and Gams (2003), propose that some *Mortierella* species in the *Mortierella isabellina* group are actually better placed in the genus *Umbelopsis*.

This indicates that these species are very closely related and that the status of some species might change with future studies. In the present study we did not reach the species level; therefore we are unable to conclude whether these two isolations could be the same species, according to this new definition. Additionally, *Umbelopsis* is a genus normally associated with root of healthy Scots pine although it has also been found in declining trees (Giordano et al. 2009).

4. Discussion

4.1 Fungal associates: are they really common associates?

The isolated species in the present study have already been found associated with *Tomicus piniperda* in previous studies in other countries (Mathiesen1950, Rennefelt 1950, Mathiesen-Käärik 1953, Lieutier et al. 1989, Piou et al. 1989 Solheim and Långström 1991, Kirisits 2000, Jankowiak 2006, Jankowiak and Bilański 2007). *Ophiostoma canum* and *O. minus* were even found in eastern Finland. In agreement with that study, *O. canum* was also more common than *O. minus* in the present study (Linnakoski 2011a). *O. canum* and *O. piceae* are indistinguishable by ITS sequence or by many morphological traits, except with the aid of specific morphological characters. *O. canum* has globose conidia (see Figure 3) that separates them from the other species and these conidia are also larger than those of *O. piceae* (Harrington et al. 2001).

Umbelopsis has been found present in decayed *Pinus* in Italy (Giordano et al. 2009), therefore it is not surprising that it was isolated from *T. piniperda* in the present study, since the insects were collected from felled or weakened trees. Zygomycetes seem to casually associate with bark beetles, therefore they are not considered as relevant or influential associates (Kirisits 2007).

The relationship between *T. piniperda* and its associated fungal community is similar to that of several other bark beetles where common associates are scarce and casual associates are abundant (Kirisits 2007, Linnakoski 2011a). More comprehensive studies in different seasons and years could be more conclusive regarding their status as associates of *T. piniperda*. However, the vast amount of previous studies where they have already been isolated from this insect seems to indicate they are in fact associated with *T. piniperda* in Finland as well as in other countries in the region.

In the present study *Leptographium wingfeldii* considered as one of the most common associates of *T. piniperda* was conspicously absent. Previous studies have

found this fungus associated to *T. piniperda* in Sweden, Germany, Poland, and even North America (Grosmann 1931 cited by Solheim and Langstrom 1991, Solheim and Långström 1991, Jacobs et al. 2004 , Jankowiak 2006, Jankowiak and Bilański 2007). This was further complicated when considering that *Sydowia polyspora* and *Ophiostoma minus* that usually occur together with *L. wingfeldii* in Sweden and France, were present in this study (Lieutier et al. 1989, Piou and Lieutier 1989, Solheim and Langstrom 1991). On the other hand, this fungus was also absent in the comprehensive study by Linnakoski (2011) in Eastern Finland. This fungus was also not present in association with *T. piniperda* in the study by Romón et al. (2007).

This could lead us to the hypothesis that *L. wingfeldii* might not be associated with *T. piniperda* in Finland. It would be convenient, nonetheless, to remember that many *Grosmannia* are the teleomorph phase of some *Leptographium* species (Zipfel et al. 2006) and that a *Grosmannia* was isolated from the Northern insects. This isolate might be *G. olivaceae* (R. Linnakoski, personal communication, March 3, 2011b) but the bad quality of the sequence did not allow us to have a definite confirmation.

Future studies could focus on finding *L. wingfeldii* associated with *T. piniperda* or another native bark beetle.

4.2 Latitude and associated mycota

The fungal population carried phoretically or internally by *T. piniperda* showed no significant difference in the North or in the South. The absence of a differentiated mycobiota in the internal or superficial body of the insects suggests that the difference of environment of the interior and surface are negligible for the fungal spores. Both environments could be equally suitable for the local fungal population to be associated with *T. piniperda*.

An interesting finding was that *O. canum* and *O. minus* were present as epi or endo-mycota, contrary to the statement of Jankowiak and Bilański (2007) that Ophiostomatoid fungi are not usually found in the surface of insects. However,

Wingfield et al. (1993) indicated that finding Ophiostomatoid fungi on the surface of insects is rather common. At the same time, *O. minus* has been registered as occurring exclusively on the surface of insects (Lombardero et al. 2003) but in the present study it was also found as endo-mycota associate. This suggests that *O. minus* can also be carried in the inside of its insect associates, at least for the case of *T. piniperda* in Finland. The question as to whether the insect's interior or surface are equally suitable for this and other Ophiostomatoid fungi merits further investigation. Other methodologies to identify whether the isolated fungi belong to endo-mycota could also be recommended.

As expected, sufficient evidence given by the statistical results and the biodiversity indices indicates that Southern mycota associates are different and more abundant than the Northern ones. Microorganisms tend to become richer as the latitude decreases (Hillebrand 2004). The sites in the North were 2.8ºC colder than the sites in the South and with 83.72% less precipitation, these differences could have influenced the mycobiota diversity. On average, the southern fungal associates per insect was a third larger than the Northern associates. Warmer conditions, such as the case of the South compared to the North of Finland, could be better for the development and diversification of some fungi (Deacon 2006). In agreement with this, latitudinal differences have been found to affect fungal abundance in Finland, where abundance was also higher in the South than in the North (Terhonen et al. 2011). In the present study the mycota isolated from *T. piniperda* was separated by 5º of latitude.

4.3 Implications of the associated fungi

Previous studies have suggested that *T. piniperda* could vector *Ophiostoma minus* and *Leptographium* spp., which are pathogenic fungi (Piou et al. 1989). Notwithstanding, in the present study *Ophiostoma canum* was the most common blue-stain fungi isolated. Even though *O. minus* was present in this study, it was very rarely isolated (2.19%) and *L. wingfeldii* was not present. Despite blue-stain fungi being considered dangerous for timber production, it has been suggested that *O.*

canum represents only a minor threat to *Pinus sylvestris* since it is less virulent than *O. minus* (Solheim et al. 2001). At the same time, *O. minus* has been found to be less virulent than *L. wingfeldii* on *Pinus halapensis* and on *Pinus brutia* (Ben Jamaa et al. 2007). Additionally, *O. minus* was isolated exclusively from the South.

The presence of *O. minus* could be related to the decrease of larval survival in *Dendroctonus frontalis*, (Lombardero et al. 2003). If that were also the case for *T. piniperda* it could be interesting to know if it is somehow related to the low association between this fungus and the insect.

Sydowia polyspora, teleomorph of *Hormonema dematiodes*, has already been found associated with *T. piniperda* (Solheim and Långström 1991). This fungus, often associated with current season needle necrosis on *Abies* spp (Talgø et al. 2010), was not very commonly isolated in this study (0.55%). This result contrasts with previous studies (Lieutier et al. 1989, Kirisits 2007, Jankowiak and Bilański 2007) that indicated that *H. dematioides* was the most commonly related to *T. piniperda*. It is also not a very virulent fungi (Lieutier et al. 1989).

Only 4 of the 26 species isolated are tree pathogens or potential tree pathogens, such as the two *Ophiostoma*, *Phoma* sp. and *Grosmannia* sp. These results agree with the statements that: only a small part of the fungal-beetle associates are relatively virulent while the other associates are less virulent, and that *T. piniperda* associates very loosely with ophiostomatoid fungi (Kirisits 2007). Under these conditions, it seems that fungal associates of *T. piniperda* might not really be providing it with an advantage to defeat tree defences, as proposed by Six and Wingfield (2011).

Beauveria bassiana, the most common isolate although found only in the northern insects, can grow as a saprotroph but also as an endophyte, in which case it could protect its host against insects (Elliot et al. 2000, Lewis et al. 2009, White et al. 2002 cited by Zhang et al. 2011). Endophytes are thought to be beneficial for their plant host because they can help activate plant defence genes. Many are also generalist invertebrate pests that help resist attacks from herbivores (Deacon 2006, Saikkonen

2007). At the same time, this fungus is suitable as a model to understand co-evolutionary processes (Saikkonen 2007) such as fungal development and host-pathogen interactions (Zhang et al. 2011).

The reasons why it was isolated only from the northern insects in this study are unknown. However, due to its ubiquitous nature (Rehner and Buckley 2005) and the previous isolations from *T. piniperda* in other studies (Humber and Hansen 2005, Jankowiak 2006, Burjanazde 2010), this finding is not new. The potential of this fungus for use in biocontrol has not been disregarded (Rehner and Buckley 2005, Lewis et al. 2009, Ormond et al. 2010, Zhang et al. 2011) and as a biocontrol agent it could be less harmful than chemical control.

It could be hypothesized that *B. bassiana* requires a colder climate, hence its absence in the South; however, it has been found in warmer countries such as France (Humber and Hansen 2005, Jankowiak 2006, Burjanazde 2010). It becomes more confusing when considering that *B. bassiana* can be an endophyte, and endophytes were expected to be more abundant in southern Finland (Terhonen et al. 2011).

Lack of appropriate temperature for sporulation and spore germination, high humidity or moisture and other microclimate and soil conditions could limit the presence of fungal entomopathogens (Deacon 2006, Wegensteiner 2007). Future studies could confirm whether this fungus is present in association with the pine shoot beetle in the area or not.

Kuraishia sp. was present in both regions, although more abundantly in the north. Different species of *Kuraishia* sp. have been found to be the teleomorph of some *Candida* spp., that usually grow on rotten wood (Péter et al. 2009). These are dimorphic fungi that grow as yeast but under different environmental conditions can switch to hyphal growth (Deacon 2006). Yeast associated with insects could help larvae in degrading complex wood components (Crowson 1984).

Trichoderma are also commonly found in the soil, but can be opportunistic and avirulent symbionts of plants or mycoparasites (Harman et al. 2004). These species are not only important for the degradation of dead insects, but also recently there has been increased interest in using them for biofuel production and granite weathering (Meeuwse et al. 2011, Brunner et al. 2011). Additionally, soil fungi like *Trichoderma* sp., *Penicillium* sp. and others, including blue-stain fungi, have also been isolated from Scots Pine in Finland (Savonmäki et al. 1992).

Further research could focus on whether the fungi associated with *Tomicus piniperda* accomplish any ecological function related to this insect and how the insect affects the patterns of fungal invasion. Other studies could also focus on whether the yeast found in this and other studies help pine shoot beetles to digest hemicellulose and other carbohydrates (Pignal et al. 1988 cited from Sauvard 2007). Many fungal spores can be found on the insects, but how many of them will actually be successful in colonizing the environment where they fall (Norros et al. 2011) could merit further research.

Therefore, in the present conditions *Tomicus piniperda* does not seem to represent a major threat as a dispersal agent of highly virulent fungi in Finnish forests.

If climate change predictions are right, a warmer climate awaits Finnish forests. It could be expected that fungal populations common in the South could progress to the North (Lindner et al. 2008). Increase in plant growth could lead to an increase in the fungal populations of an area (Rogers 2011). According to the results in the present study, few fungal associates in the South are pathogenic and most of the isolated fungi are non virulent. This could indicate that even under a warmer climate these fungus-insect interactions are not a potential threat. However, trees might be affected by the outbreak of other pests in Fennoscandic forests and thus become more susceptible to the attack of this insect (Lidner et al. 2008). Additionally, fungal population of some bark beetles can switch to more pathogenic associates during outbreaks (Viiri and Lieutier 2004). Nevertheless, an outbreak of this insect might not even occur, since pine shoot beetles are affected by other factors besides

temperature that inhibit the occurrence of more than one generation per year (Sauvard 2007).

4.4 Technical issues

4.4.1 Sampling methodology

The sampling method for this experiment was subjective sampling, considering that *Tomicus piniperda* mostly affects weakened pines (Annila et al. 1999). Therefore, targeted sampling areas where weakened pines that have been suffering stress by fire, pollution or fungi attack or trees that have been recently felled can be more effective than isolation from healthy trees. *T. piniperda* responds to monoterpentes in the resin of low vigour Pine trees, and it prefers those trees than high vigour individuals (Byers 2007). Other studies have also collected samples from weakened or recently felled trees (Jankowiak and Bilanski 2007, Linnakoski et al. 2011).

Studies report that ethanol used as bait inhibits attraction of *T. piniperda* (Klimetzek et al. 1986, Schroeder 1988) but a contradictory result was found by Byers (2007). Although pheromone traps have been used for *T. piniperda*, trap trees or trap logs are more common for its collection (Grégoire and Evans 2007).

An important evidence of the presence of the insect that helped in its collection was the reddish-brown frass left by the female when it penetrates the wood to form the galleries (see Figure 2). Females form the gallery for the future larvae, while the male removes the debris from the tunnel (PA IPM 2010) this can be used for the localization and collection of the insect.

4.4.2 Sterilization

Several factors seem to point to the fact that the sterilization process may not be optimal. One of them is the high presence of molds in the study. The other is the lack of difference between epi- and endo-mycota populations. Yet another is the presence

of *O. minus* as endo-mycota. Morphotypes identified as yeast were placed in a specific media, but molds were also observed growing in them. This could have been caused by contamination of the agar by handling procedures.
The procedure of placing the insect directly in the agar could be questionable, however other studies have also placed insects without sterilization on agar (Piou et al. 1989, Jankowiak and Bilański 2007). Future research should use better sterilization method to avoid growth of molds, while specific media can also be used to avoid these and bacteria. However *Penicillium* and other molds have been found as common associates of *T. piniperda* (Mathiesen-Käärik 1953, Jankowiak 2006, Jankowiak and Bilański 2007), which validates the results of this study.

4.4.3 Length and limitations of the study

Recent studies on mycota population associated with insects are a result of more than one year of work and repeated collections in different seasons (Piou et al. 1989, Jankowiak and Bilanski 2007, Linnakoski 2011a) rather than a collection of one group of samples done in one year. The fact that the present study was limited by collection in one season could have influenced the results and shown only a fraction of the commonly associated fungi of *T. piniperda*. However, it is a well known fact that most of the associates of the pine shoot beetle and other beetles are casual (Kirisits 2007, Six and Wingfield 2011). Thus, due to this behaviour it could have occurred that under the conditions of the collection sites the association did not occur with many of these fungi. Nevertheless, the amount of fungi found in the present study is more than one fourth of that found by Jankowiak and Bilanski (2007). Mackenzie and Royle (2005) indicate that collecting more often on fewer sites could be a better use of the resources and will give a more precise estimation of the species present in a determined area.

Another matter to be considered is the methodology of cultivation, morphotyping and sequencing of fungi. Similar morphotypes that represent different species could be confused as one, therefore a portion of the fungal species could remain undetected (Maia 2011).

The use of ITS region for identification has some issues since the ITS region could be insufficiently conserved or even confused by introgression or biparental inheritance patterns (Buchheim et al. 2011). These and other problems related to the use of ITS regions can be overcome using a sequence-structure approach and using the regions of variability and substantially conserved areas found analysing the secondary structure (Coleman 2003, Coleman 2007, Schulz et al. 2005, Schulz and Wolf 2009, Keller et al. 2010).

Additionally, fastidious fungi, obligate biotrophs and slow-growing fungi usually escape detection in this type of study (Fröhlich and Hyde 1999, Beebee and Rowe 2004, Rogers 2011, Terhonen et al. 2011). Therefore other molecular methods such as the pyrosequencing and the use of molecular markers can give a broader result and help clarify genus or species status (Nilson et al 2009, Linnakoski 2011a). However, the use of ITS region complemented with morphological characteristics give strength and validity to the present study.

5. Conclusions

Knowledge of beetle-fungus interactions is still scanty and there are several gaps to be filled. One of the aims of this study was to identify the potential fungal associates of *Tomicus piniperda* L. The other was to find if the population of these varied from North to South. The results of this work suggest that a difference exists between the fungal population associated with *Tomicus piniperda* in the South and the North of Finland. Another finding was that there were no significant differences between the mycota associated with the insect carried phoretically or internally.

Due to the high population of moulds isolated from the insects in the different sites and with different treatments, it can be inferred that the methodology for sterilization was not optimal. This is supported by the results of the similarity index, where the highest similar population was that of the South epi- and endo-mycota groups of fungi. Other techniques to isolate endo-mycota could clarify whether the difference in surface and interior parts of the insects affect the fungal population.

The fungal species isolated from *T. piniperda* in this study are mostly common isolates that have been reported in other countries as well (Mathiesen1950, Rennefelt 1950, Mathiesen-Käärik 1953, Lieutier et al. 1989, Piou et al. 1989 Solheim and Långström 1991, Kirisits 2000, Jankowiak 2006, Jankowiak and Bilański 2007). Very few tree pathogens were isolated: *Ophiostoma canum*, *O. minus*, *Grosmannia* sp. and *Sydowia polyspora*. *O. canum* was the most commonly isolated among these species and it has been suggested that it is less virulent than *O. minus* (Solheim et al. 2001) and therefore it poses no great threat. *S. polyspora* is only an opportunistic pathogen of conifers and mostly found as endophyte in pines. Such results could suggest that this particular insect is associated mainly with avirulent fungi in Finland. Nevertheless, other studies should confirm if the same occurs when it becomes an alien species in a foreign environment, such as North America.

Saprotrophic fungi such as *Trichoderma* and *Penicillium* have also been reported in other studies as associated with this particular bark beetle (Lieutier et al. 1989,

Jankowiak 2006, Jankowiak and Bilański (2007). Such coincidences might indicate that it is probable that the mentioned species are common associates of *T. piniperda.* However, more studies collecting *T. piniperda* in different seasons and years could help to confirm their status as either common or casual associates.

The lack of Basidiomycota in the identified isolates in the present study supports similar results noted in other studies (Linnakoski 2010). It could potentially indicate that in the regions studied, the beetles do not associate with Basidiomycota or that the methodology used for isolation is not appropriate to promote the growth of these fungi. A proportion of the fungal population present in this study is likely to remain undetected, whether because they are not culturable, are obligate biotrophs, or seasonal transients, among other reasons (Fröhlich and Hyde 1999).

Further studies using other diverse sampling approaches or the latest metagenomics next generation sequencing method could help to shade more light on fungal diversity associated with this bark beetle. Taking into account these results for the purposes of climate change related fungal-insect association alterations in the pine shoot beetle in Northern Finland, it can be assumed that these associates would not represent a big threat. Despite this, if climate change causes an outbreak of this insect, it could associate with other, maybe more pathogenic, fungi (Viiri and Lieutier 2004). Changing fungal populations related to the size of the insect population could be a topic for future studies.

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Appendices

Appendix 1: Reagents

List of solutions for DNA extraction

Preparation For 13 ml 2% CTAB

2% (w/v) CTAB, pH 8

1M Tris-HCl

0.5 M EDTA, pH 8

M NaCl

2% (v/v) βmercaptoetanol

Preparation of TE buffer 10:1

1M TRIS-HCl, pH 8

0.5M EDTA, pH 8

List of reagents for PCR

15 µL twice autoclaved MQ water

2.5 µL 10x buffer

0.5 µL 200 µM dNTP

0.5 µL 0.5 µM ITS-1F

0.5 µL 0.5 µM ITS-4B

0.5 µL 0.2 U/µL Polymerase

0.5 µL 1mM MgCl2

List of reagents and solutions for agarose gel electrophoresis

Agarose gel

0.5g Agarose gel (1%)

50 ml 1X TAE buffer

2 µL Ethidium bromide

Appendix 2: Figures

Figure S1: Larvae of *Tomicus piniperda* L.

Figure S2: Collection site in the South, targeted sampling site is a tree recently affected by snow.

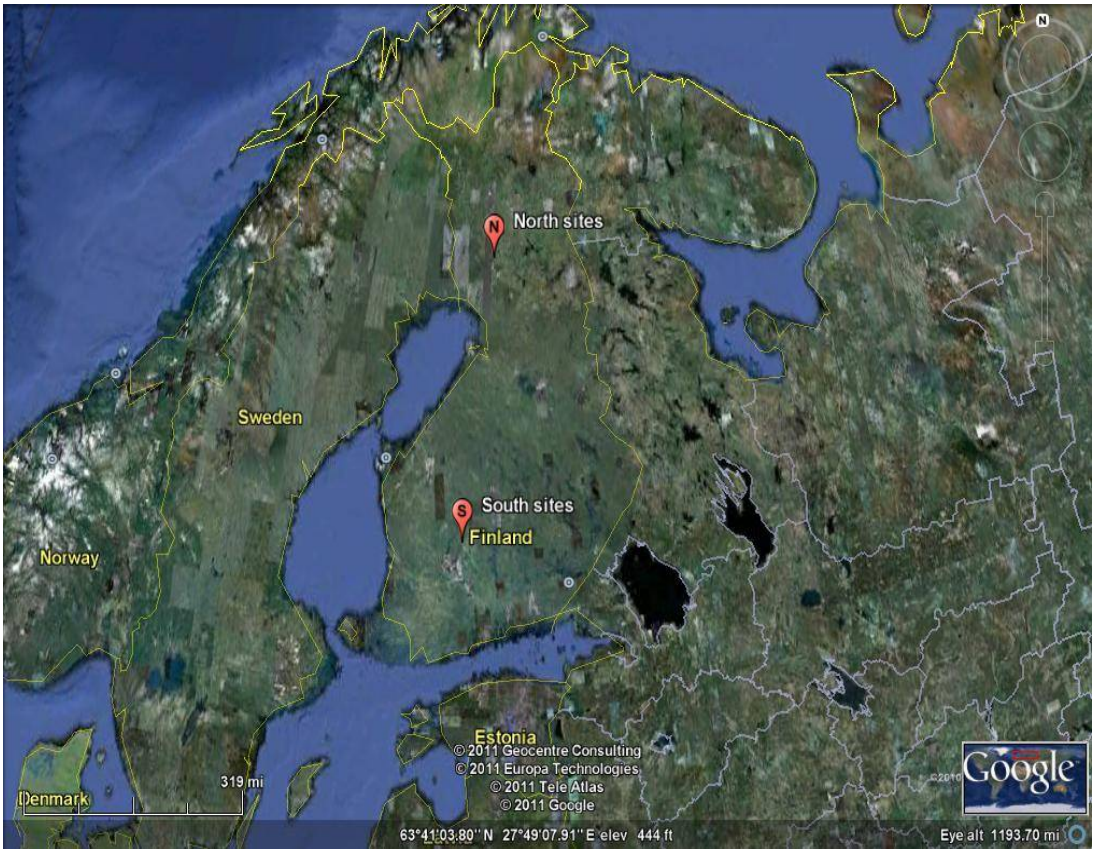

Figure S3: Collection sites of *Tomicus piniperda* in the North and in the South of Finland.

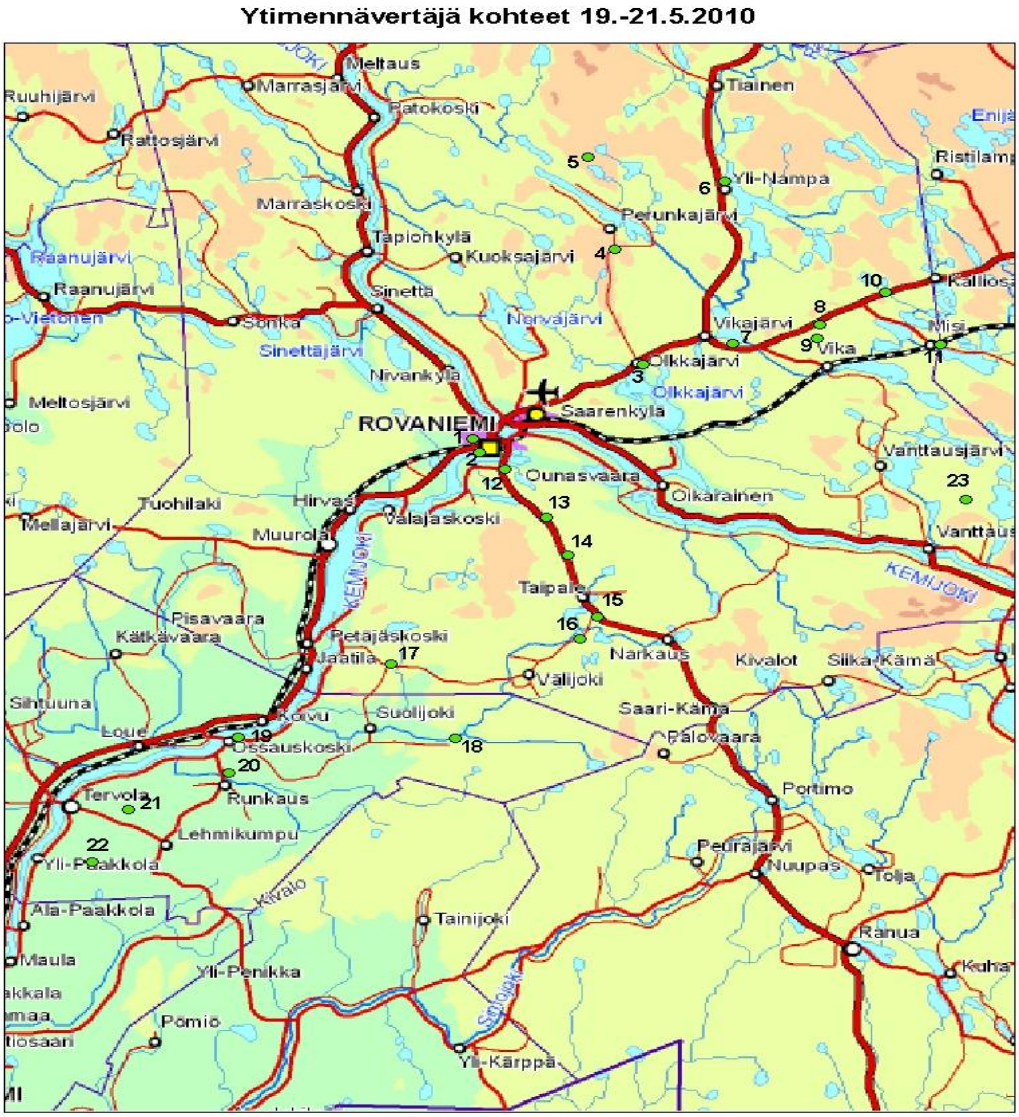

Figure S4: Collection sites of *Tomicus piniperda* in the North.

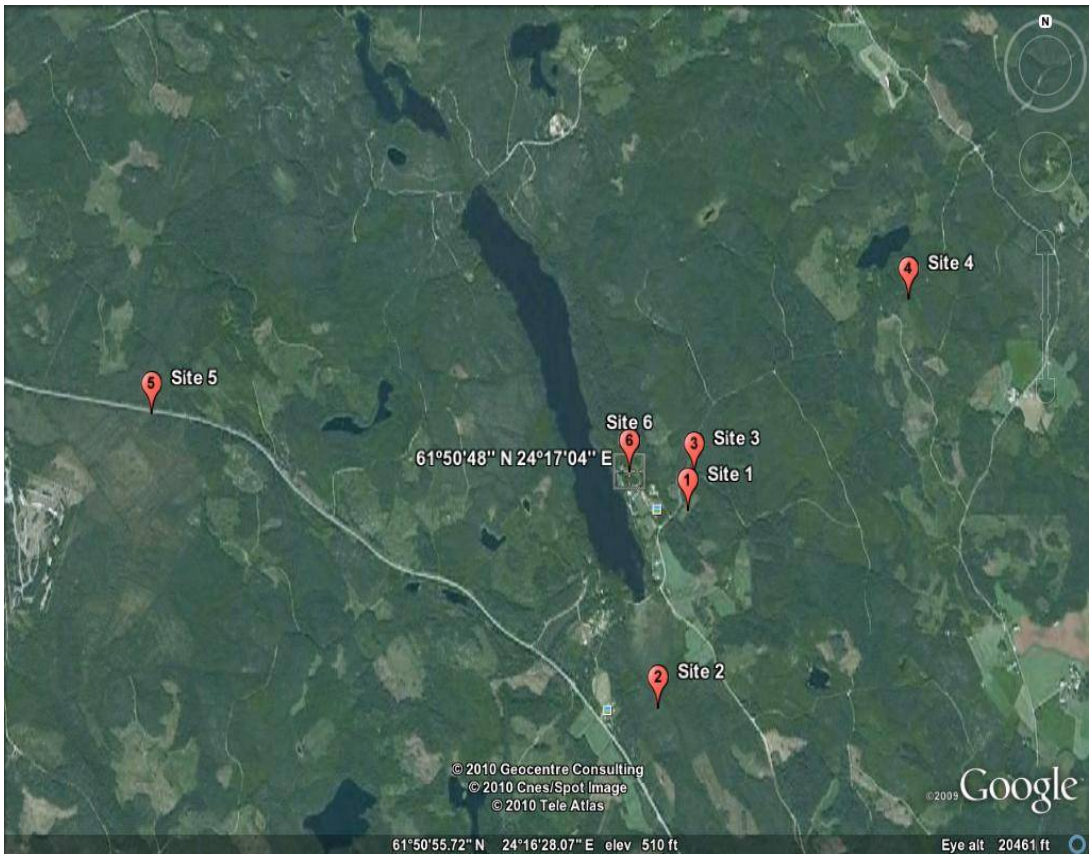

Figure S5: Collection sites of *T. piniperda* in the South.

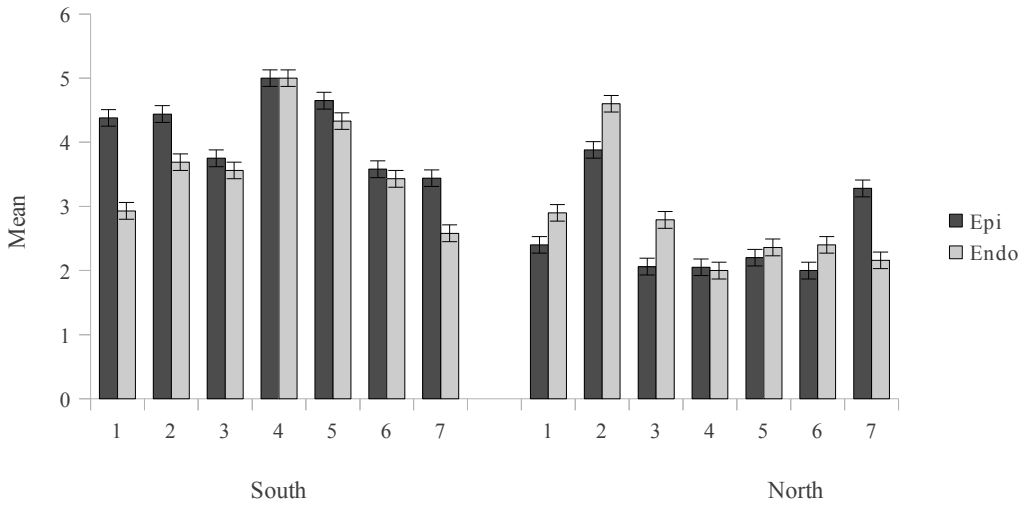

Figure S6: Mean fungal population per site per geographical location. Significant differences were observed between northern and southern population.

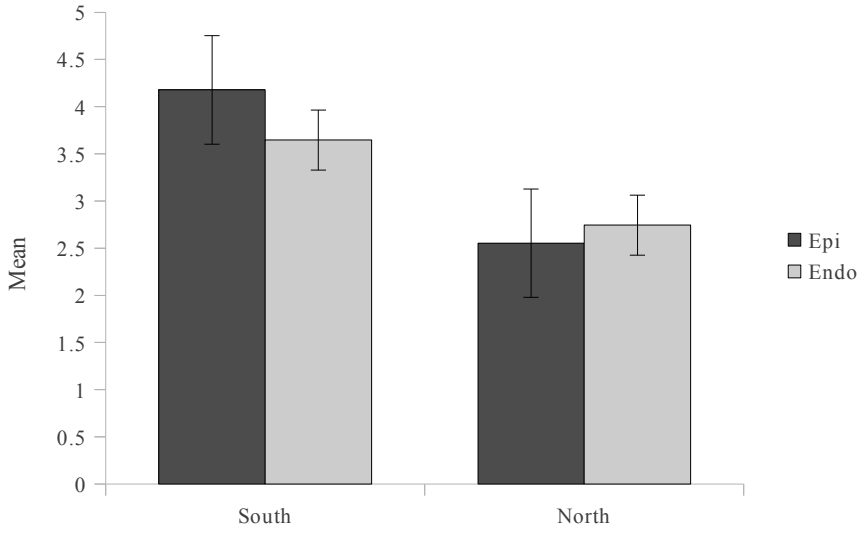

Figure S7: Mean fungal population per insect location. Significant differences were observed between northern and southern population.

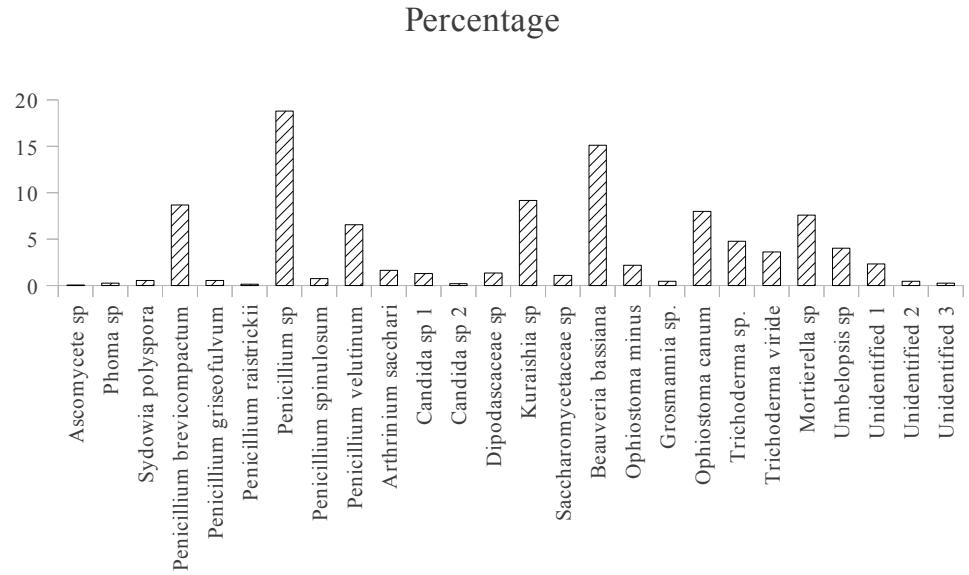

Figure S8: Percentages of total fungal isolates from seven sites in the North and seven sites in the South.

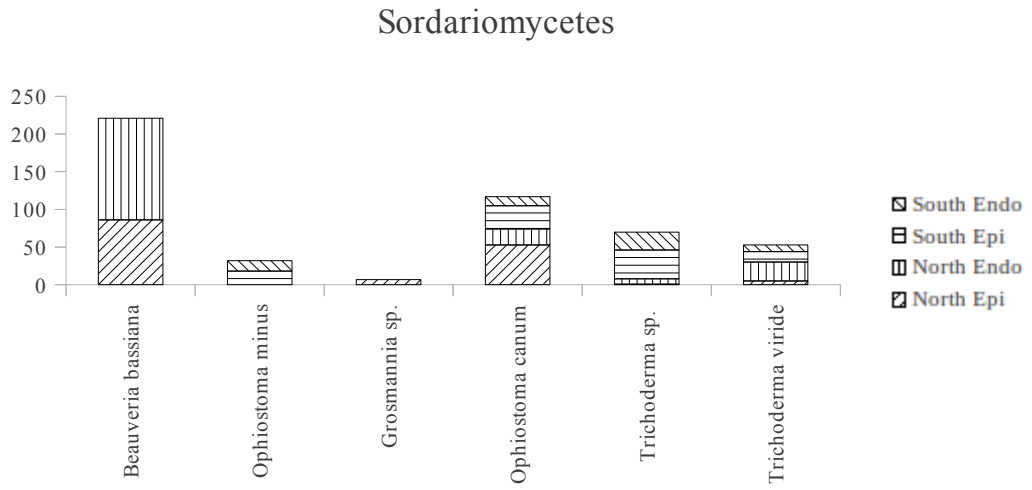

Figure S9: Number of isolated Sordariomycetes from South and North.

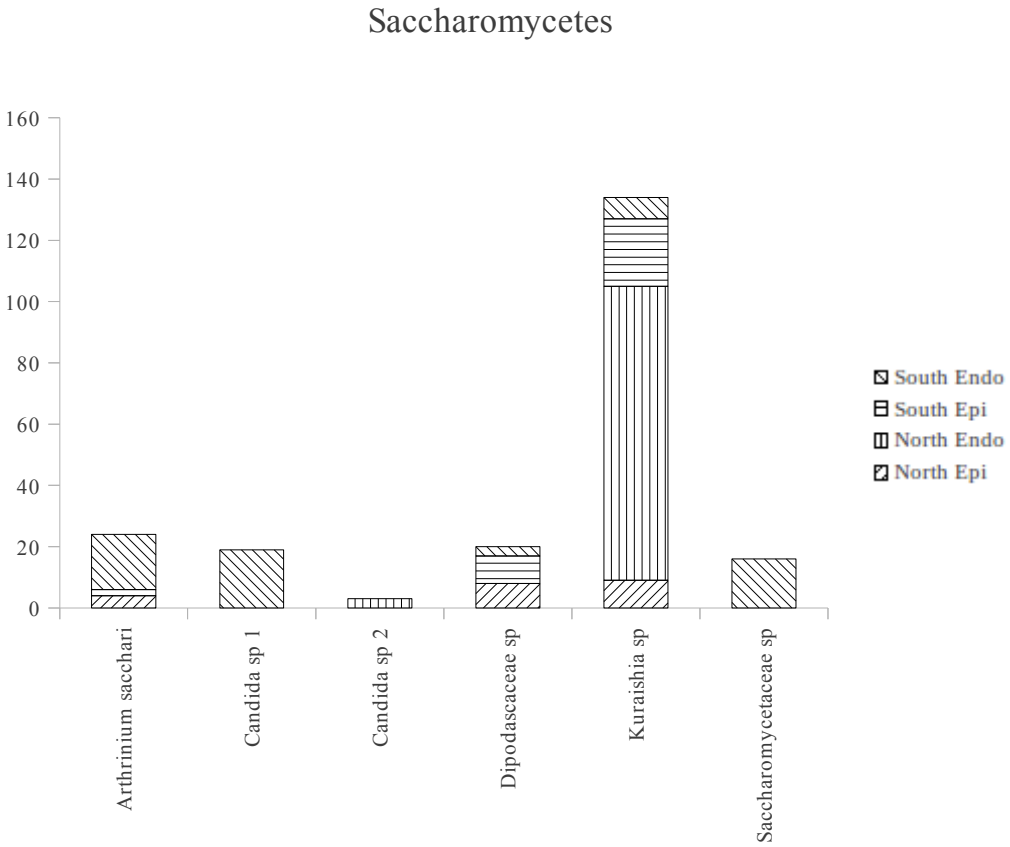

Figure S10: Number of isolated Saccharomycetes from North and South.